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Effects of Low-Dose Bisphenol A on DNA Damage and Proliferation of Breast Cells: The Role of c-Myc

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Abstract

Background: Humans are exposed to low dose Bisphenol A (BPA) through plastic consumer products and dental sealants with BPA. While a number of studies have investigated the mammary gland effects after high-dose BPA exposure, the study findings differ. Furthermore, there has been a lack of mechanistic studies.

Objective: The objective of this study is to investigate the effect and the mechanism of low dose BPA in mammary gland cells.

Methods: We evaluated DNA damage following BPA exposure using the comet assay and immunofluorescence staining, and used cell counting and three-dimensional cultures to evaluate effects on proliferation. We examined the expressions of markers of DNA damage and cell-cycle regulators by immunoblotting and performed siRNA-mediated gene silencing to determine the role of c-Myc in regulating BPA's effects.

Results: Low-dose BPA significantly promoted DNA damage, upregulated c-Myc and other cell-cycle regulatory proteins, and induced proliferation in parallel in estrogen receptor- α (ER α)-negative mammary cells. Silencing c-Myc diminished these BPA-induced cellular events, suggesting that c-Myc is essential for regulating effects of BPA on DNA damage and proliferation in mammary cells.

Conclusions: Low-dose BPA exerted c-Myc-dependent genotoxic and mitogenic effects on ER α -negative mammary cells. These findings provide significant evidence of adverse effects of low-dose BPA on mammary cells.

Introduction

BPA is widely found in a number of household products, including food and water containers, linings of metal food and beverage cans and dental fillings (Rochester 2013). BPA is routinely detected in human urine, blood, and breast milk due to low but continuous exposure (Lassen et al. 2014; Peretz et al. 2014; Yi et al. 2013). Nine out of ten Americans tested positive with BPA in random urine samples (Lassen et al. 2014). In a study measuring BPA in breast adipose tissue, more than half of the examined samples had detectable BPA levels (Fenichel et al. 2013).

BPA has no structural homology with 17 β -estradiol (E2), but because it is similar to Diethylstilbestrol (DES), the synthetic estrogen known to cause cancer (Kurosawa et al. 2002), BPA has long been suspected to be able to induce carcinogenesis (Keri et al. 2007). Rodents exposed to environmentally relevant BPA levels around the time of birth showed characteristics associated with an increased risk for developing breast cancer (Ayyanan et al. 2011; Munoz-de-Toro et al. 2005). However, the mechanism by which BPA exerts its biological actions is still obscure. Although the effects of high-dose BPA on proliferation in ER α -positive cells have been studied (Olsen et al. 2003; Samuelsen et al. 2001), the molecular mechanisms underlying low-dose BPA-mediated regulation of proliferation and DNA damage in ER α -negative breast cells are largely unknown. Moreover, considering that a majority of adult human breast tissues expresses low levels of ER α (Boyd et al. 1996), non-estrogenic mechanisms of action for BPA need to be elucidated.

One mechanism that has been suggested to explain BPA toxicity in DNA damage is the production of reactive oxygen species (ROS) (Kovacic 2010). However, it remains unclear whether BPA at low (nanomolar) doses can induce ROS and DNA damage in ER α -negative

mammary cells. DNA double-strand breaks (DSB) are the most harmful damage to the genome integrity among the various types of damage to DNA (Rothkamm and Löbrich 2003). Ataxia-telangiectasia mutated (ATM) is activated through its autophosphorylation at Ser-1981 (ATM-pS1981). This activation of ATM induces phosphorylation of its downstream targets histone H2A variant H2AX at Ser-139 (γ -H2AX), another marker for DSB and DNA damage (Bakkenist and Kastan 2004). In addition to the ATM-mediated DNA damage response, it has been found that upregulation of oncogenic c-Myc induces ROS and promotes DNA damage (Vafa et al. 2002). Moreover, c-Myc upregulation suppresses repair of DNA DSB (Li et al. 2012) that can lead to oncogene-induced genetic instability, which is an evolving hallmark of cancer (Negrini et al. 2010). In parallel, deregulated c-Myc can also debilitate p53-mediated cell cycle arrest (Vafa et al. 2002) and promote proliferative signaling, which is a hallmark of cancer (Hanahan and Weinberg 2011). However, it is unknown if low-dose of BPA can act as a DNA damaging agent to induce phosphorylation of these DNA damage-associated proteins and to promote upregulation of c-Myc in mammary cells.

Studies on the mitogenic, apoptotic, and transcriptional properties of BPA are inconsistent (Dairkee et al. 2008; Diel et al. 2002). The inconsistency has been attributed to wide variation in doses and the high (micromolar range) doses that are often used (Lapensee et al. 2009). Studies of cellular effects on mammary cells after low, environmentally relevant doses of BPA are needed. A study conducted by the US Centers for Disease Control and Prevention had detected BPA in 95% of urine samples from a reference population of 394 American adults (Calafat et al. 2005). This study reported average levels of total BPA in male and female urine of 1.63 and 1.12 ng/ml, respectively, suggesting lower exposures in women compared with men. Based on more than 80 published human biomonitoring studies, it has been estimated that unconjugated BPA

was routinely detected in blood in the range of nanograms (ng) per milliliter (ml), which is similar to the range of conjugated BPA detected in the urine samples (Vandenberg et al. 2010). It has been estimated, based on measurable levels of BPA in body fluids and tissues, that the theoretical internal dose of the general population is 10-100 nM (Vandenberg et al. 2007). Therefore, we chose to study the effects of environmentally relevant doses of BPA in the nanomolar range (10-100 nM). In discussions on BPA safety, it is mentioned that evidence at the basic molecular level is often missing (Kovacic 2010). Therefore, we sought in this study to identify the molecular mechanisms mediating the effect of BPA on mammary cells.

In this study, we examined the effects of low dose BPA exposure on breast cell lines at the cellular and the molecular level to understand the mechanism(s) by which BPA leads to mammary gland effects. We also examined the ability of BPA to regulate the levels of proteins associated with DNA damage and proteins controlling proliferation in these cells at low doses. Here we show that low-dose BPA independently promotes DNA damage and proliferation and that c-Myc upregulation is a key mechanism by which BPA exerts its DNA-damaging and proliferative effects on ER α -negative mammary cells.

Materials and Methods

Materials

Materials and reagents used in these analyses are listed in Supplemental Material, Chemicals and antibodies.

Cell lines and siRNA transfection

The MCF10A (ER α -negative) and 184A1 (ER α -negative) human cell lines are the immortalized benign and normal breast epithelial cell lines, respectively. The MCF7 (ER α -positive) and

MDA-MB-231 (ER α -negative) cell lines originate from human breast epithelial adenocarcinomas. All these cell lines were obtained from ATCC. Details regarding cell culture conditions are summarized in Supplemental Material, Cell culture conditions. For transfection with siRNA, specific siRNA against c-Myc (sc-29226) and control siRNA (sc-44231) were obtained from Santa Cruz Biotechnology. MCF10A and 184A1 cells were transfected with the c-Myc siRNA or control siRNA by using DharmaFECT 1 transfection reagent (Thermo Scientific) as described previously (Chung et al. 2012).

Western blotting (immunoblotting)

Cells were washed twice in phosphate-buffered saline (PBS) and then lysed with a lysis buffer containing protease inhibitors for 20 min on ice. Whole cell lysates were centrifuged at 10,000g for 15 min to remove cell debris. Protein concentration of lysates was determined with Bio-Rad Protein Assay. Cell lysates (30 μ g protein/each) were electrophoresed onto sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis gels. The separated proteins were transferred to nitrocellulose membranes (Bio-Rad) before blocking with 5% bovine serum albumin (BSA) in Tris buffered saline (TBS) with 0.1% Tween-20 (Sigma). Membranes were incubated with primary and then horse-radish peroxidase-conjugated secondary antibodies, in blocking buffer. The immunoblots were visualized on film with the West-Q chemiluminescence kit (GenDEPOT). β -actin represents the loading controls.

Immunofluorescence

Cells were seeded on round coverslips, and were treated with EtOH or BPA 24 hr after seeding. The cells were washed in PBS, fixed in 4% paraformaldehyde for 15 min at 4°C, and washed in PBS again before permeabilization with a PBS containing 0.1% Triton-X (Sigma). The

coverslips were blocked in 1% BSA in permeabilization buffer for 1 hr at room temperature and incubated overnight with specific antibodies as described (Chung et al. 2012). After washing, the coverslips were incubated with an Alexa-488-conjugated secondary antibody for 1 hr and the nuclei were stained with DAPI solution (Sigma) for 10 min. After washing, the coverslips were mounted on glass slides and observed under a Leica-TCS-SP2 Laser Scanning Spectral Confocal Microscope. Images were acquired using the Leica-SP2 software. A cell with more than 5 staining foci was considered positive.

The comet assay

To determine the level of DNA damage in 184A1, cells were treated with EtOH or BPA for 3 or 24 hr and were subjected to a comet assay to detect DNA damage and repair at the level of single cells under neutral conditions as described previously (Tsai et al. 2008). Briefly, after treatment, cells were harvested and mixed with low-melting temperature agarose. After lysis, electrophoresis was performed at 1 V/cm and 15 mA for 40 min. Slides were stained with SYBR Green dye for 10 min. One hundred randomly selected cells per sample were captured under a Zeiss fluorescent microscope and digital fluorescent images were obtained using the AxioVision software. The relative length and intensity of SYBR Green–stained DNA tails to heads is proportional to the amount of DNA damage present in the individual nuclei and is measured by Olive tail moment using TriTek Comet Score software.

Analyses of intracellular ROS levels

Cells were seeded on round coverslips in 12-well plates, treated for 2 or 24 hr with EtOH or BPA, and subsequently incubated with 50 μ M DCF-DA and MitoTracker (Invitrogen) at 37°C in the dark for 30 min. Cells were then fixed with 4% paraformaldehyde, washed 3 times with PBS,

counterstained with DAPI, and washed 3 times again with PBS. The coverslips were mounted on glass slides and observed under a Confocal Microscope, and images were acquired as described above.

Cell proliferation assays

Cell proliferation was evaluated by cell counting assay and the MTT assay as described previously (Park et al. 2014). For cell counting assay, cells were seeded in a 6-well plate at 25,000 cells/well in 5 replicates for each treatment. Following treatment, the number of cells was counted in duplicate in a hemacytometer using 0.4% Trypan Blue. For the MTT assay, 2000-4000 cells were seeded into 96-well plates. At the end of the treatment, cells were incubated with 200µl 2.5% 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) for 2 hr, and the absorbance was determined at 560 nm colorimetrically using an ELISA plate-reader. Cell proliferation was calculated as the ratio of the absorbance from treated samples compared with that of control sample.

Three-dimensional (3D) culture

Cells were seeded in eight-well chamber slides (Lab-Tek™ II, Thermo Scientific) (5000 cells/well), treated with EtOH or BPA, and 3D-spheroid assays were performed as described previously (Hu et al. 2014). The wells were first coated with 60 or 120 µl of Extracellular Matrix (ECM) Gel from Engelbreth-Holm-Swarm murine sarcoma (Sigma). The cells were seeded on top of the gel layer in DMEM/F12 medium for MCF10A with 2.5% ECM gel and 5 ng/ml EGF. Cells were treated 24 hr after initial seeding. Cells were grown for 14 days with the change of the top layer media with drugs every 4 days. Analysis of the spheroid size and calculation of the spheroid number were performed as described (Hu et al. 2014).

Statistical analysis

All data are expressed as means and standard deviations (SD) from at least three determinations. The statistical significance of difference between two groups were analyzed with two-sided unpaired Student's *t*-tests when the variances were equal with Grahpad PRISM statistical software or Excel. P values less than 0.05 were considered statistically significant.

Results

Effects of low-dose BPA on H2AX and ATM phosphorylation and γ -H2AX and ATM-pS1981 positive breast cells

Because phosphorylation of DNA damage-associated proteins (ATM and H2AX) is a major indicator of DSB, we examined whether a low dose (10 nM) of BPA induces the levels of ATM-pS1981 and γ -H2AX with time course experiments in 184A1, MCF10A, MCF7 and MDA-MB-231 breast cells. By using immunoblotting, we demonstrated that both ATM-pS1981 and γ -H2AX were indeed upregulated when cells were exposed to BPA at 10 nM (Supplemental Material, Figure S1A-D).

With immunofluorescence staining, foci of γ -H2AX can be seen at the points of DSB. To determine the effect of low doses of BPA on DNA damage, we analyzed the number of cells with γ -H2AX positive nuclei after treatment with EtOH, 100 nM E2, 10 nM BPA, or 100 nM BPA. All studied cell lines had a greater number of positive nuclei after treatment with BPA than the control for 3 hr (Supplemental Material, Figures S1E,F) or 24 hr (Figure 1A-D; Supplemental Material, Figures S2A-D). The number of positive cells ranged from 40-60%, with the highest number of positive cells seen in the normal-like 184A1. Phosphorylation of ATM at Ser-1981 was also investigated. Both MCF10A and MCF7 had a greater number of nuclei positive for

ATM-pS1981 after treatment with BPA than the control for 24 hr (Supplemental Material, Figure S2E-H). These data suggest that low-dose BPA may induce DSB in breast cells.

Effects on c-Myc and other cell-cycle regulatory proteins

It has been shown that one mechanism of DNA-damage induction in cells is through upregulation of the c-Myc oncogenic protein (Vafa et al. 2002). Hence, we sought to determine whether low-dose BPA could upregulate c-Myc in ER α -negative breast cells. By using immunoblotting, we show that low-dose BPA (10 nM) significantly increases the level of c-Myc protein in a time-dependent manner in MCF10A and 184A1 cells (Figure 1E,F). Since c-Myc upregulation can increase cell proliferation rate (Schmidt 1999), we examined whether BPA could regulate the expression of other oncogenic cell-cycle regulatory proteins such as cyclin D1, cyclin E, and E2F1 in these cells under the same conditions. Similarly, our data show that low-dose BPA increases the levels of cyclin D1, cyclin E, and E2F1 in mammary cells (Figure 1E,F). However, the negative control EtOH does not alter the levels of these cell-cycle regulatory proteins in the treated cells (Supplemental Material, Figure S3A). Similarly, our data show that BPA (10 nM) upregulates the expression of these cell-cycle regulatory proteins in a time-dependent manner in MCF-7 [wild-type (wt)-p53] cells (Supplemental Material, Figure S3B).

To test whether c-Myc is required for BPA-promoted increase of γ -H2AX, MCF10A and 184A1 cells were transfected with c-Myc siRNA or control siRNA for 48 hr, and c-Myc knockdown cells and control cells (Supplemental Material, Figure S3C,D) were treated with EtOH or BPA for 24 hr and the levels of γ -H2AX were analyzed. Our results indicate that silencing c-Myc reduces BPA-mediated increase of γ -H2AX (Figure 1G-J), suggesting that c-Myc plays an essential role in BPA-induced DNA damage.

DNA damage

Under neutral conditions, the comet assay directly correlates DNA DSB in individual cells with the relative tail moment, which is defined as the product of the distance between the head and the center of gravity of DNA in the tail and the percentage of DNA in the comet tail. The results of the comet assay illustrated staining of the DNA from individual cells from 184A1 after treatment with EtOH or 100 nM BPA. After 3-hr exposure to BPA, a significant increase of olive tail moment was seen, showing that BPA induces rapid DSB in genomic DNA of normal mammary cells (Figure 2A,B) that is consistent with rapid formation of γ -H2AX foci (Supplemental Material, Figure S1E,F).

To examine whether c-Myc is essential for BPA-induced DSB and whether both DSB and γ -H2AX foci take place at the same BPA exposure time (24 hr), c-Myc knockdown 184A1 cells and control cells were treated with EtOH or BPA for 24 hr and the levels of comet tail moment were analyzed. Our results show that silencing c-Myc significantly reduces BPA-mediated increase of the comet tail moment (Figure 2C,D), whose incubation time is consistent with that for γ -H2AX foci (Figure 1A-D). These results suggest that c-Myc is necessary for BPA-induced DSB.

ROS production

Because a major mechanism of DNA-damage induction is through oxidative stress, we utilized the fluorescent probe DCF-DA to determine whether low-dose BPA could induce ROS in breast cells. Figure 3A,B illustrate a rapid induction of ROS in 184A1 and MCF7, respectively. Each was treated with 10 nM and 100 nM BPA, with an increase in staining intensity with higher dose. The ROS staining was localized to the mitochondria as shown by the colocalization with

Mitotracker staining, whose incubation time is consistent with that for H2AX phosphorylation (γ -H2AX) (Supplemental Material, Figure S4).

To assess whether c-Myc is required for BPA-induced ROS production, c-Myc knockdown MCF10A cells and control cells were treated with EtOH or BPA for 24 hr and the levels of production of ROS were analyzed. Our results indicate that silencing c-Myc abolishes BPA-mediated ROS production (Figure 3C,D), whose incubation time is consistent with that for γ -H2AX foci in Figure 1G-J. These results suggest that c-Myc is essential for BPA-promoted ROS production.

Proliferation of mammary cell lines

An important question in the signaling mechanism is how low-dose BPA contributes to the increase of proliferation in ER α -negative noncancerous breast cells. It has been shown that the c-Myc oncogenic protein can induce proliferation and transform mammary epithelial cells (Cowling et al. 2007). Thus, using the standard cell proliferation assays, we investigated whether low-dose BPA could induce proliferation in various breast cell lines. As shown in Figure 4A,B, low-dose BPA can induce accelerated proliferation markedly in the two ER α -negative wt-p53 mammary cell lines. In contrast, E2 (100 nM) shows no significant effect on these cell lines. For a comparison, we tested whether low-dose BPA could induce growth in breast cancer cell lines. As shown in Figure 4C,D, low-dose BPA potently augments proliferation in wt-p53 MCF7 cells, whereas the effect of low-dose BPA on inducing proliferation is greatly diminished in the mutant-p53 cell line MDA-MB-231. Similar effects were seen with E2.

To examine whether the effect illustrated from BPA could be explained by an upregulation of ER α , the expression of ER α was examined by immunoblotting in these cells after treatment with

EtOH or BPA for 72 hr. Our data show that low-dose BPA exerts no effect on ER α expression in the treated cell lines (Figure 4E). These results confirm that low-dose BPA promote proliferation in mammary cells in an ER α -independent manner.

To examine whether c-Myc is required for BPA-induced proliferation, c-Myc knockdown MCF10A and 184A1 cells and their control cells were treated with EtOH or BPA for 48 hr and the levels of proliferation were determined. These results indicate that silencing c-Myc significantly decreases BPA-mediated proliferation (Figure 4F,G), suggesting that c-Myc is necessary for BPA-promoted proliferation.

Proliferation of mammary cells in 3D culture

The mammary gland is composed of multiple cell types and they are surrounded by an ECM. Together, they form complex interactions necessary for the mammary gland's development and function (Polyak and Kalluri 2010). The conventional breast cell assays in 2D cell culture cannot mimic changes and interactions in authentic breast tissue and tumor microenvironments, whose distinctive 3D structures are critical for breast cancer development *in vivo*. Thus, to examine whether low-dose BPA would have the same proliferative effect on ER α -negative breast cells in a more tissue-like microenvironment, MCF10A cells were grown in an ECM gel in 3D spheroids. The size of spheroids was measured to determine whether the cells treated with low-dose BPA grew faster than the cells treated with ethanol (controls). Cells that were grown in 100 nM BPA had a higher median area and had a higher percentage of spheroids 2-, 3- and 4-times larger than the cutoff value than controls (Figure 5A-C). Treatment of these cells with 100 nM E2 seemed to have a negative effect on proliferation in 3D culture.

To test whether c-Myc is required for BPA-induced spheroid proliferation, c-Myc knockdown MCF10A cells and control cells were treated with EtOH or BPA and the levels of spheroid proliferation were determined. Our results indicate that silencing c-Myc significantly reduces BPA-mediated spheroid proliferation (Figure 5D-F), suggesting that c-Myc plays an essential role in BPA-promoted spheroid proliferation.

Effect of c-Myc silencing on BPA-induced cell-cycle regulatory proteins

To examine whether c-Myc is essential for BPA-mediated upregulation of the expression of cell-cycle regulatory proteins, c-Myc knockdown MCF10A and 184A1 cells and their control cells were treated with EtOH or BPA and the levels of the specific cell-cycle proteins were analyzed. These data show that silencing c-Myc markedly decreases the BPA-induced expression of the specific proteins (Figure 6A,B), suggesting that c-Myc is necessary for BPA-promoted oncogenic cell-cycle regulatory proteins. Collectively, our results suggest that low-dose BPA can induce the expression of oncogenic cell-cycle regulatory proteins leading to the increase of proliferation in ER α -negative mammary cells in a c-Myc-dependent manner.

Discussion

In this study, we examined the effects of low concentrations of BPA (nanomolar doses) on DNA damage, the expression of oncogenic cell-cycle regulatory proteins, and on the proliferation in normal-like, noncancerous, and cancerous breast cells. We have shown that (a) BPA, at environmentally relevant doses, has a genotoxic effect on mammary cells; (b) BPA upregulates the expression of c-Myc and other crucial oncogenic cell-cycle regulatory proteins in these cells; (c) BPA causes the induction of ROS in these cells; (d) BPA has a mitogenic effect by increasing the rate of proliferation in wt-p53 breast cells; (e) c-Myc is required for all these cellular events

induced by BPA. To our knowledge, this is the first discovery showing that low-dose BPA upregulates c-Myc, which induces DNA damage and proliferation in ER α -negative mammary cells.

Previous studies have shown that high doses of BPA were cytotoxic, but that exposure to BPA at low, noncytotoxic levels did not induce genotoxic effects (Audebert et al. 2011). Our results showed that BPA at noncytotoxic levels did induce genotoxic effects. In the study by Audebert et al, cells were only treated for 24 hr, while in this study cells were treated with BPA for up to 72 hr and in some cell lines the DNA damaging effects were seen as late as 24-48 hr. Considering that BPA is thought a possible weak carcinogen (Cavalieri and Rogan 2010) and that the general population is exposed to a low chronic dose (Vandenberg et al. 2007), it is of interest to study longer exposure times as well.

When mice were injected with ER α -dependent MCF7 cells and also treated with either high-dose BPA or a placebo, tumors were found in those treated with BPA (Weber Lozada and Keri 2011). We studied the low-dose effect of BPA on DNA damage and proliferation in various mammary cell lines, and showed that BPA induced DNA-damage markers in cells regardless of the ER α status. To our knowledge, this is the first study showing low-dose BPA (nanomolar doses) in regulating non-estrogenic proliferation. We found that in the ER α -negative cells, low-dose BPA did increase proliferation, while E2 did not. In MCF-7, however, both E2 and BPA increased proliferation. Although a higher dose of BPA usually displays a greater effect on proliferation in ER $^{+}$ breast cancer cells, our BPA dose-dependent findings suggest that a higher dose of BPA may not exert a greater effect on proliferation in normal breast cells (184A1) (Figure 4B,G). These findings suggest that the BPA dose-dependent effects on ER $^{+}$ breast cancer cells may be distinct

from those on ER⁺ normal mammary cells. Moreover, BPA did not induce ER α expression in ER α -negative breast cells. Our results confirm that the observed proliferative effects of BPA on these cells cannot be attributed to a potential upregulation of ER α expression. Moreover, BPA has been shown to have the same antagonistic effect on anticancer drugs regardless of the ER α status of breast cells (Lapensee et al. 2009). Taken together, our data as well as other studies suggest that BPA can act on mammary cells through ER α -independent mechanisms.

It has been shown that high doses of BPA can induce ERK1/2 via GPR30 in breast cancer cells (Dong et al. 2011). In this study, we showed that when ER α -negative mammary cells were exposed to low-dose BPA, the expression of important oncogenic cell-cycle regulatory proteins, including c-Myc, cyclin D1, cyclin E, and E2F1, was significantly induced. It has been established that c-Myc increases proliferation rate and oncogenic transformation (Cowling et al. 2007; Schmidt 1999). Notably, upregulating c-Myc also promotes DNA damage and induces ROS in cells (Vafa et al. 2002). These findings are consistent with our results that low-dose exposures of BPA can induce c-Myc, increase ROS and cause DNA damage, as well as promote proliferation in mammary cells. However, the possible mechanisms for BPA induction of ROS remain unclear. It has been reported that upregulation of the Ras oncogene and its downstream effectors such as NADPH oxidases (NOXs) can induce ROS (Ogrunc et al. 2014). Thus, it's possible that BPA-mediated upregulation of the c-Myc oncogene may promote NOXs and increase ROS in breast cells.

Recently, some additional pathways have been suggested to be involved in the adverse effects after BPA exposure. For instance, while BPA exposure induces activation of the mTOR pathway (Akt1, RPS6, pRPS6 and 4EBP1), BPA reduces the p53 proapoptotic pathway (p53, p21WAF1

and BAX) in human breast epithelial cells, resulting in increasing proliferation initiating gene products (including proliferating cell nuclear antigen, cyclins, cyclin-dependent kinases, and phosphorylated pRb) (Dairkee et al. 2013). Thus, the effects of these additional pathways after BPA exposure can be tested to further build an understanding of the adverse outcome pathway for BPA exposure and mammary gland cellular effects.

In proliferation assays, our results indicate that low-dose BPA potentially promoted proliferation in all three wt-p53 breast cell lines (Figure 4A-C), whereas the effect of low-dose BPA on inducing proliferation was decreased in the mutant-p53 MDA-MB-231 (Figure 4D). These results suggest that the effect of BPA on inducing proliferation may depend on p53 function. In contrast, low-dose BPA potentially induced DNA damage markers, γ -H2AX and ATM-pS1981, in all four breast cell lines (i.e., MCF10A, 184A1, MCF7, and MDA-MB-231) (Supplemental Material, Figures S1, S2; Figure 1A-D). These results suggest that BPA may induce DNA damage in MDA-MB-231 cells via a p53-independent mechanism.

Although low-dose BPA potentially induced key DNA damage markers in MDA-MB-231 (Supplemental Material, Figure S1D), low-dose BPA was unable to induce proliferation significantly in MDA-MB-231 (Figure 4D). These data strongly suggest that there is no causal relationship between DNA damage and proliferation. In fact, it has been documented that DNA damage induces cell cycle checkpoints, which lead to either cell cycle arrest for DNA break repair or inducing cellular apoptosis (Kastan and Bartek 2004; Zhou and Elledge 2000). Thus, DNA damage should not cause proliferation directly in normal breast cells, which have functional cell cycle checkpoints and the DNA repair activity. Moreover, our data indicate that

low-dose BPA considerably upregulates the expression of c-Myc (Figure 1E,F; Figure 6A,B) in mammary cells.

Although the ultimate goal is to extrapolate *in vitro* findings to human health, it is premature to do so. Further studies of the effect of low-dose BPA on mammary gland carcinogenesis and cancer development in the rodent models are needed to validate the effect of low-dose BPA on an *in vivo* situation, which may contribute to new insight into this health problem.

Further investigation of the role of low-dose BPA in regulating the global gene-expression changes and the genome-wide epigenetic alternations in mammary cells would aid in assessing linkages between low-dose BPA exposure and cell proliferation, DNA damage, mammary gland transformation, and carcinogenesis. Understanding the molecular basis of potential effects of low-dose BPA on mammary carcinogenesis may suggest innovative preventive modalities to improve human health. Also, at least in theory, benefits of understanding the mechanism could extend beyond preventive measures targeted only at BPA (such as phthalates, parabens, and other environmental chemicals).

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Figure Legends

Figure 1. Low-dose BPA induces nuclear γ -H2AX levels through c-Myc. (A) MCF10A cells were treated with ethanol (EtOH) or BPA (10 or 100 nM) for 24 hr. The levels and subcellular localization of endogenous γ -H2AX were detected using an anti- γ -H2AX antibody, followed by an Alexa-Fluor-488-conjugated secondary antibody. DAPI was used to visualize the nuclei. (B) An average of 150 of the stained cells was analyzed and a histogram shows the percentage of cells with nuclei positive for γ -H2AX. Similarly, (C, D) 184A1 cells were treated with EtOH or BPA for 24 hr, the levels of γ -H2AX were detected, and the histograms were displayed. **, $P < 0.0005$. (E) MCF10A and (F) 184A1 cells were treated with BPA (10 nM) for a time course. Total lysates from these cells were subjected to Western blotting using specific antibodies. (G, H) MCF10A and (I, J) 184A1 cells were transfected with c-Myc-siRNA or control-siRNA for 48 hr, and c-Myc-knockdown and control cells were treated with EtOH or BPA for 24 hr, the levels of γ -H2AX were detected, and the histograms were shown. Scale bar: 20 μ m.

Figure 2. Low-dose BPA induces DNA DSB in normal mammary cells through c-Myc. (A) 184A1 cells were treated with BPA or EtOH for 3 hr, and the comet assay was done under neutral conditions. (B) The relative comet tail moments represent the averages of 100 cells in three independent experiments. Similarly, (C, D) 184A1 cells were transfected with c-Myc siRNA or control siRNA for 48 hr, and c-Myc-knockdown and control cells were treated with EtOH or BPA for 24 hr, the comet assay was performed, and the relative comet tail moments were displayed. Scale bar: 40 μ m.

Figure 3. Low-dose BPA induces production of ROS in breast cells through c-Myc. (A) 184A1 and (B) MCF7 cells were treated with BPA or EtOH for 2 hr. Cells were stained with DCF-DA (10 μ M) to show ROS-induced oxidation (green) and MitoTracker-Red (50 nM), a mitochondrial dye. DAPI was used to visualize the nuclei. Merged images show the co-localization of mitochondria and ROS production. Similarly, (C, D) MCF10A cells were transfected with c-Myc siRNA or control siRNA for 48 hr, and c-Myc-knockdown and control cells were treated with EtOH or BPA for 24 hr, and ROS production was detected and shown. Scale bar: 20 μ m.

Figure 4. Low-dose BPA induces proliferation in wt-p53 breast cells through c-Myc. Wt-p53 (A) MCF10A, (B) 184A1, (C) MCF-7, and (D) mutant-p53 MDA-MB-231 cells were treated with EtOH (-), estradiol (E2) or BPA for 4 days, then trypsinized, mixed with trypan blue and counted in 5 replicates. The histograms show relative number of cells (mean \pm SD) in each sample compared with the control. (E) To show that the proliferation was not caused by an effect of BPA on upregulating ER α , Western blotting was performed using an anti-ER α antibody. The samples have been treated with EtOH or 10 nM BPA for 72 hr. The blot was stripped and re-probed for total proteins and β -Actin. (F) MCF10A and (G) 184A1 cells were transfected with c-Myc siRNA or control siRNA for 24 hr, and c-Myc-knockdown and control cells were treated with EtOH or BPA for 48 hr, relative number of cells were determined, and the histograms were displayed.

Figure 5. Low-dose BPA promotes spheroid proliferation in 3D cultures through c-Myc. Cells were incubated for 10 days in 3D ECM-gel overlay cultures under treatment with EtOH, E2 or BPA. Cells were analyzed in a bright-field microscope and the area of each spheroid above a cutoff size was measured. (A) Phase contrast/brightfield images at day 8 are shown, with all images at the same magnification, resolution and size. (B) The histogram shows the average size of spheroids (mean \pm SD, n=100). $P=0.004$. (C) Also, the percentage of spheroids that were 2, 3, and 4 times larger than the cutoff size is shown (mean \pm SD of triplicate) as shown in (B). (D-F) MCF10A cells were transfected with c-Myc siRNA or control siRNA for 24 hr, and c-Myc-knockdown and control cells were treated with EtOH or BPA for 10 days in 3D cultures, images for spheroids were displayed, and the histograms were shown. Scale bar: 100 μ m.

Figure 6. Silencing c-Myc reduces the BPA-promoted expression of cell-cycle regulatory proteins. (A) MCF10A and (B) 184A1 cells were transfected with control siRNA or c-Myc siRNA for 48 hr, and c-Myc-knockdown and control cells were treated with BPA for 24 hr, and whole lysates from these cells were subjected to Western blotting with specific antibodies.

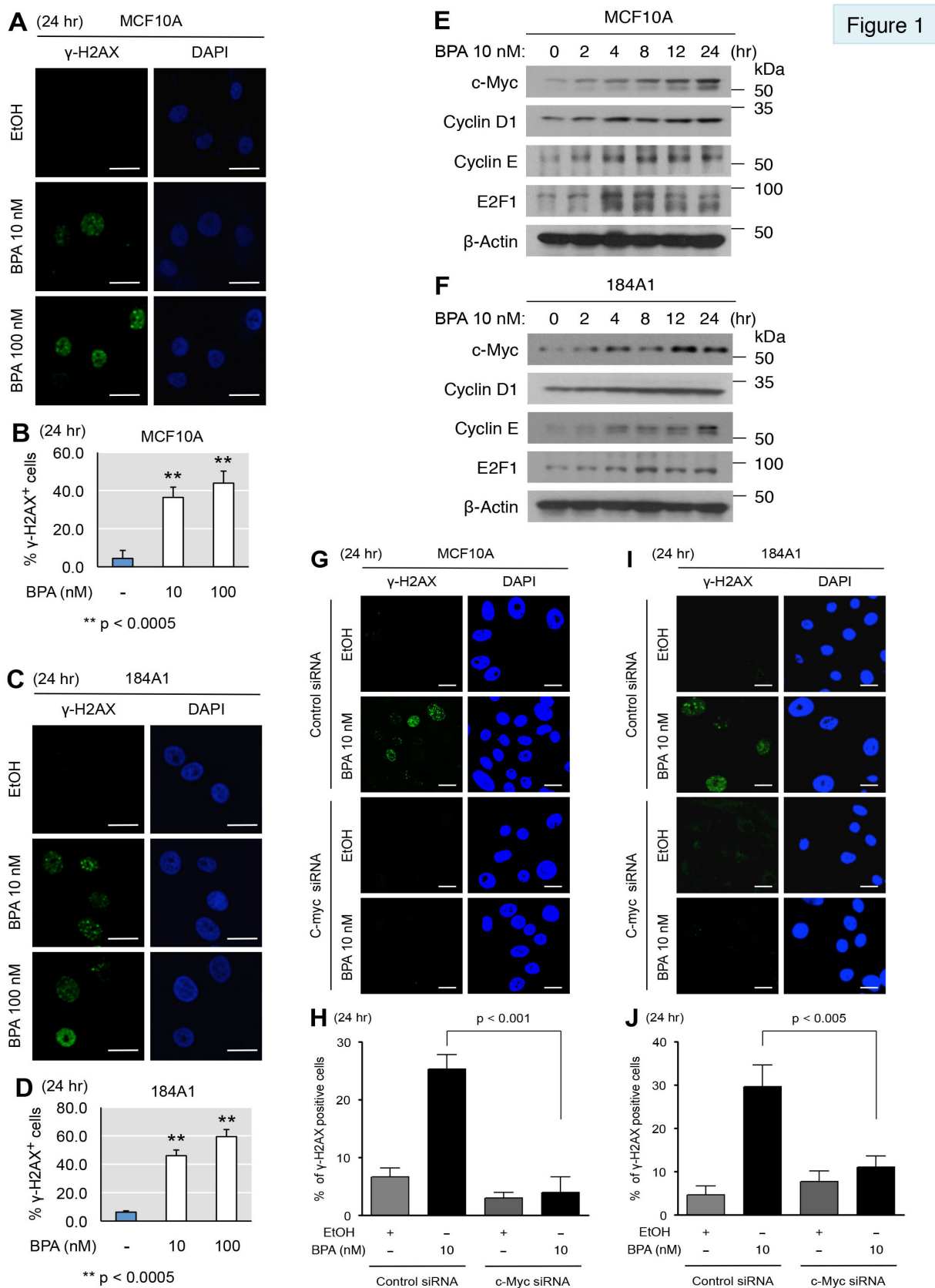


Figure 1

Figure 2

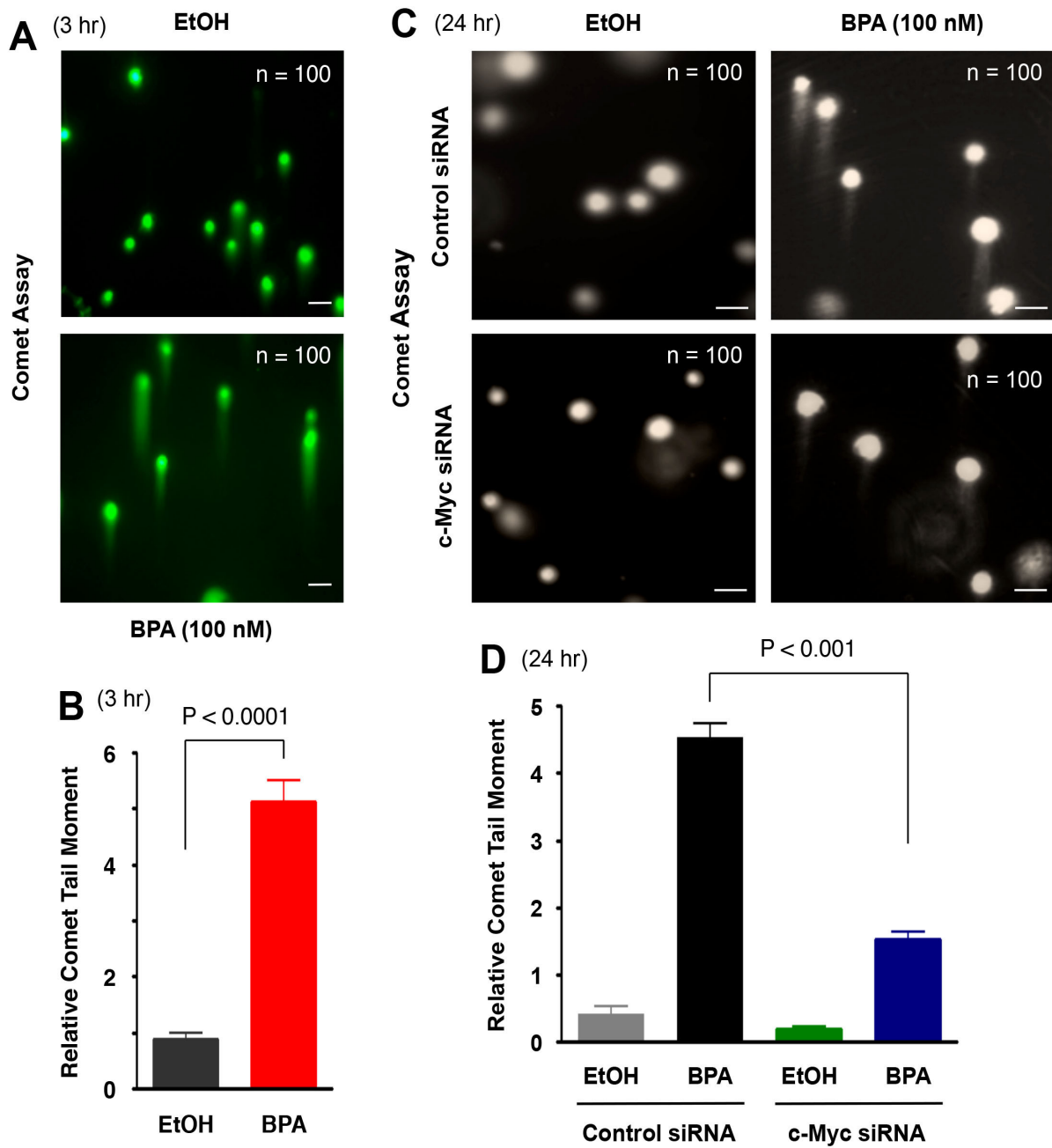


Figure 3

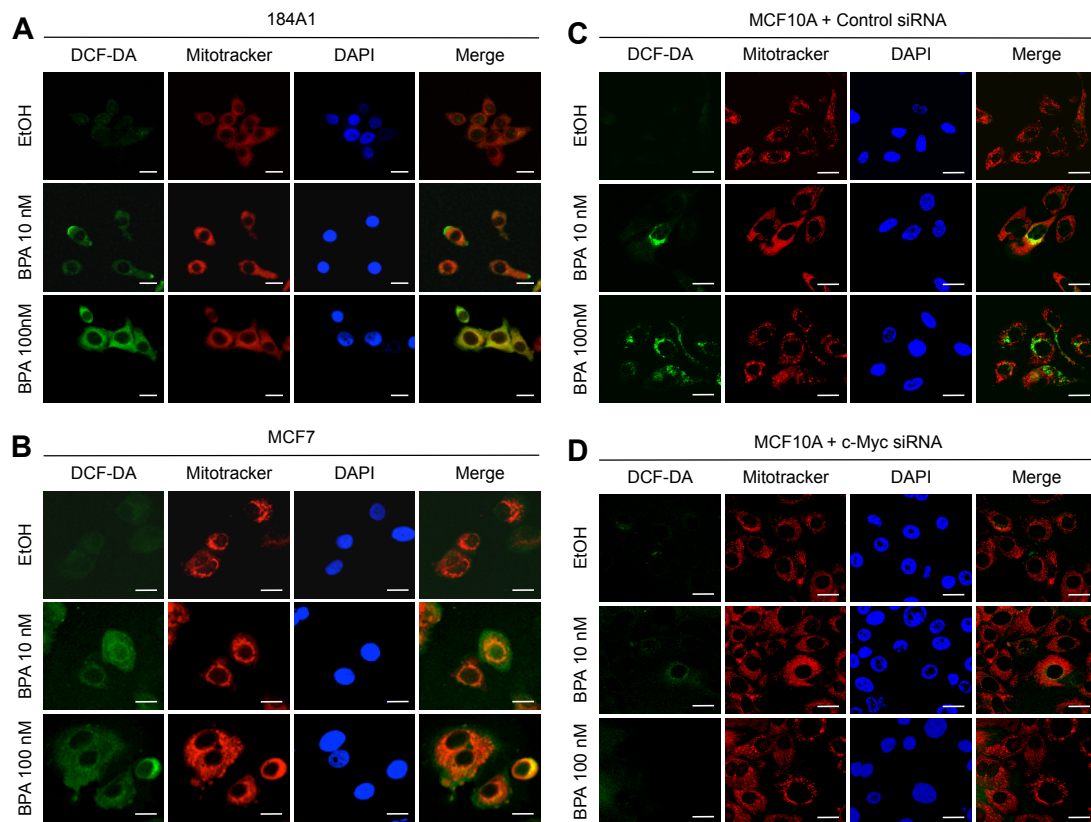


Figure 4

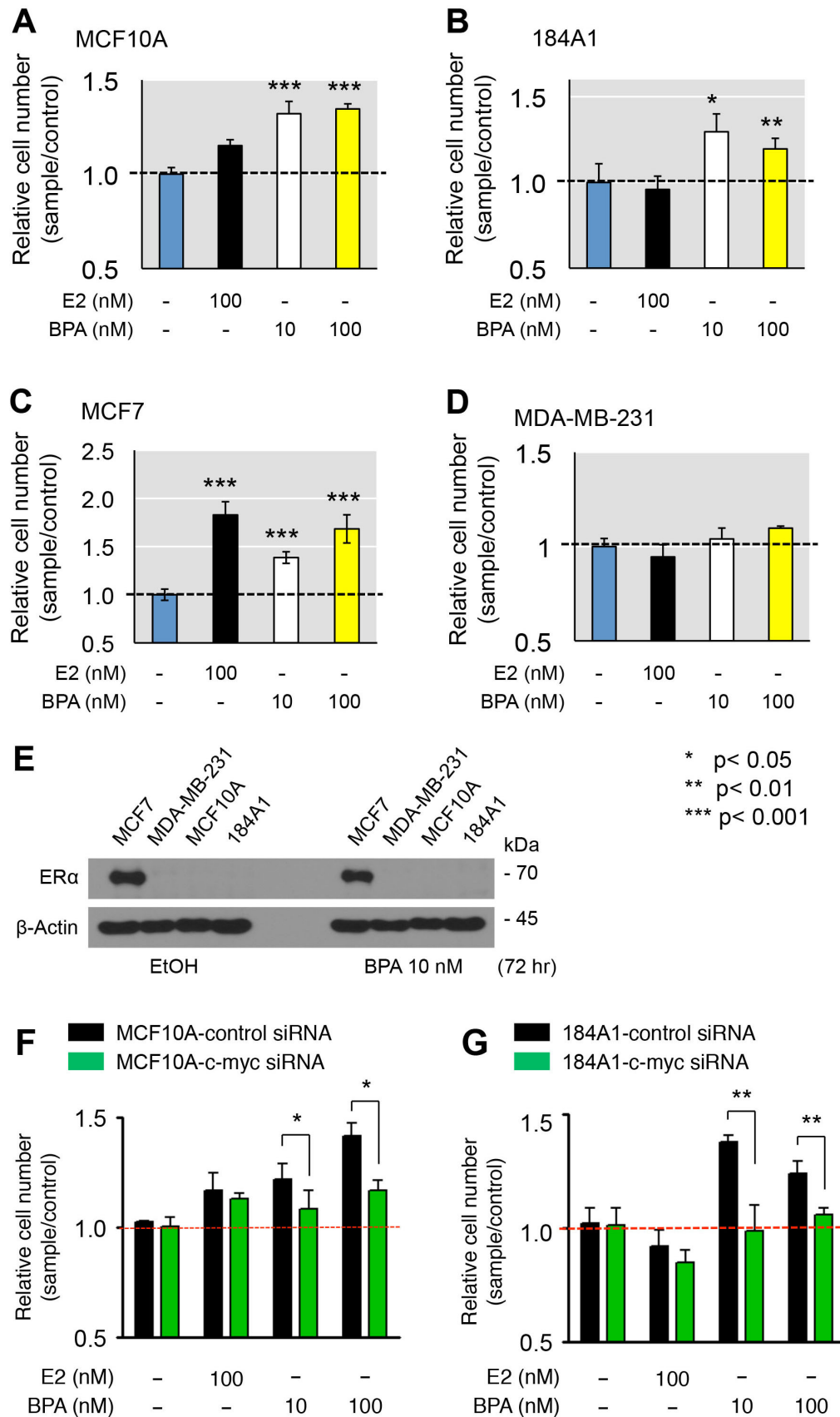


Figure 5

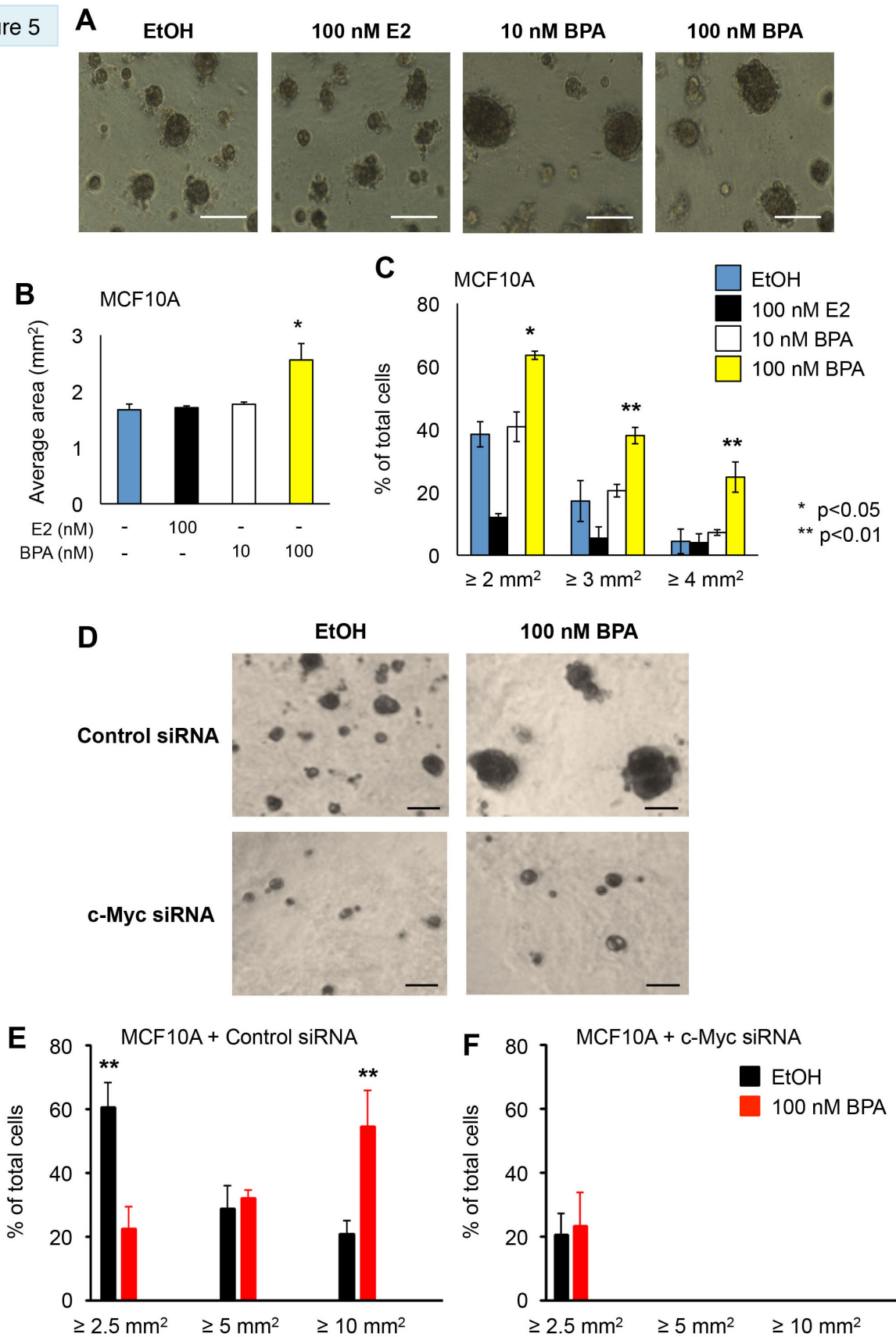


Figure 6

